A novel variant fibrinogen, deletion of Bβ111Ser in coiled-coil region, affecting fibrin lateral aggregation

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Abstract

[Background] Functional fibrinogen concentration of a male infant showed < 0.50 g/L and we speculated this patient as a dysfibrinogenemia or hypofibrinogenemia. [Methods] We analyzed propositus and his parent by DNA sequencing and by thrombin-catalyzed fibrin polymerization for purified plasma fibrinogen. [Results] Although functional fibrinogen determinations based on Clauss method showed the marked discrepancy of values among three sets of reagent and analyzer, we found a novel heterozygous variant fibrinogen, Kyoto IV, causing by 3-bp deletion in Bβ-chain gene corresponding to the deletion of 111Ser located in coiled-coil region. We guess discrepancy of fibrinogen values among three assays was caused by the difference in NaCl concentration in reagents for determination and analyzed the polymerization under the conditions of various NaCl concentrations. Although under normal physiological conditions Kyoto IV fibrinogen augmented the polymerization as compared with normal control, in 0.21 M NaCl Kyoto IV fibrinogen showed abruptly impaired polymerization curve compared with normal control. [Conclusion] The present study indicates that variant fibrinogen, BβΔ111Ser, showed augmented lateral aggregation under normal physiological conditions and the residue located in coiled-coil region, Bβ111Ser, plays an important role in the lateral aggregation.

Key Words: variant fibrinogen, fibrinogen Bβ-chain, coiled coil region, amino acid deletion

Introduction

Fibrinogen is a dimeric plasma glycoprotein, each half of the dimer being composed of three polypeptide chains (Aα, Bβ, and γ), and it is expressed as a trinodular structure formed from two distal D nodules, a central E nodule, and two coiled-coil regions linking the D and E nodules [1]. Recently, new insight into the structure of the coiled-coil region has been obtained through crystallographic studies [2-4]. This region has a three-stranded unique α-helical structure composed of Aα50Arg-160Ser, Bβ80Glu-192Tyr, and γ24Gly-134Glu residues [2-5] and is delimited at both ends by interconnected braces of cysteines in all three of the chains called "disulfide rings". The N-terminal disulfide ring is formed via three disulfide bonds: Aα45Cys-γ23Cys, Aα49Cys-Bβ76Cys, and Bβ80Cys-γ19Cys, and the C-terminal disulfide ring is also formed via three disulfide bonds: Aα161Cys-γ139Cys, Aα165Cys-Bβ193Cys, and Bβ197Cys-γ135Cys [2-5]. Furthermore, the X-ray structure of fragment D from human fibrinogen revealed that the Aα-chain makes an abrupt reversal within the distal disulfide ring, the returning chain forming a fourth helix running in the opposite direction with respect to the three-stranded coil [2]. This antiparallel Aα-chain continues to be associated with the coiled-coil at least to Aα192 and turns away from the main coiled-coil adjacent to Aα92, Bβ125, and γ60 [3,4]. Although the regular periodicity of hydrophilic and hydrophobic residues is necessary for the α-helical coiled-coil structure, the function of this region in fibrin polymerization is not well known.

By the end of 2004, 246 families with dysfunctional fibrinogen had been analyzed genetically and/or structurally. They are listed in the homepage (http://www.geht.org/databaseang/fibrinogen/). Most of these variants display amino acid substitution either in the Aα-chain (31 variants and 143 families) or in the γ-chain (37 variants and 73 families), while variants with substituted residues in the Bβ-chain numbered 19 variants in 30 families. In the present study, we report a novel and interesting variant with deletion of Bβ111Ser (BβΔ111Ser) in the coiled-coil region, termed Kyoto IV, which is characterized by augmented lateral aggregation under normal physiological conditions but by impaired fibrin polymerization in buffer at high NaCl concentration. Furthermore, we compare the polymerization of BβΔ111Ser

with six dysfunctional fibrinogens located in the coiled-coil region [6-14] and discuss the function of this region for converting of fibrinogen into fibrin.

Materials and methods

Kyoto IV patient data

The propositus was a very low birth weight male infant (1272g) who was delivered by 28 weeks and 6 days and due to fetal distress. Although the propositus showed no bleeding or thrombotic events, the coagulation test results at his birth were the followings; PT:14.5 sec (58%) (normal range: 10.2-13.4 sec, 66-115 %), APTT:63.4 sec (normal range: 26.2-39.3), functional fibrinogen concentration: < 0.50 g/L (normal range: 1.6-3.7), D-dimer: 4.4 μg/ml (normal range: < 0.9), AT-III: 29% (normal range: 70-130) (all parameters measured in Clinical Laboratory, Kyoto University Hospital). Blood was collected from the propositus (2 months after birth) and his mother and father (both with no bleeding or thrombotic events) with informed consent for biochemical and genetic analyses.

Coagulation screening tests and purification of plasma fibrinogen

After citrated-plasma had been separated from the blood of the propositus and his mother and father, coagulation screening tests were performed using an automated analyzer, MDA-II (Hitachi High Technology, Inc., Tokyo, Japan) using reagents purchased from Japan Biomeriuex (Tokyo, Japan) (for fibrinogen determination by the thrombin-time method; MDA Fibriquik). Functional fibrinogen concentrations by the thrombin-time method were also estimated using an automated analyzer, Coagulex-800 (Shimazu; Kyoto, Japan), using Dade Thrombin Reagent (Dade Berhring Marburg GmbH; Marburg, Germany) (Shinshu University Hospital) or using Thrombocheck Fib-L (Sysmex) (Kyoto University Hospital). The molarity of Na^+ or Cl^- in each thrombin reagent was determined by the ion selective electrode method and the molarity of NaCl in the reagents was estimated based on the lower molarity of both parameters. The immunologically determined fibrinogen concentration was measured as previously described [15]. The purification of fibrinogen from the plasma of the propositus' mother and the normal control was performed by a modified immunoaffinity-chromatography procedure utilizing IF-1 monoclonal antibody, as

previously described [16]. Eluted fibrinogen was dialyzed against 20 mM N-[2-hydroxyethyl] piperazine-N'-[2- ethanesulfonic acid] (HEPES), pH 7.4, containing 0.12 M NaCl. Fibrinogen concentrations were determined from the $\Delta A_{280\text{-}320}$ value, on the assumption that 10 mg/ml would give a value of 15.1 [17]. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (10% polyacrylamide gel).

Polymerase chain reaction (PCR)-amplification of the fibrinogen gene and DNA sequencing

DNA was extracted from blood cells using a DNA Extractor WB Kit (Wako Pure Chemical Ltd., Osaka, Japan). To amplify all exons and exon-intron boundaries in the Aα-chain, Bβ-chain and γ-chain genes, 32 PCR primers were designed and the DNA was amplified by PCR as described elsewhere [18]. The PCR products were purified from agarose gels and directly sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 310 Genetic Analyzer (both from Applied Biosystems, Foster City, CA). To verify the nucleotide-deletion detected by direct sequencing, the PCR product was subcloned into pCR2.1 plasmid vector (TA Cloning Kit, In Vitrogen, San Diego, CA) and sequenced as described above.

Thrombin-catalyzed fibrin polymerization

Polymerization was followed by measuring turbidity changes with time at 350 nm using a UV-110-02 spectrophotometer (Shimadzu Co., Tokyo, Japan). The reactions were performed in triplicate for each sample as described elsewhere [16,19], with a few minor modifications. Briefly, the final concentration of NaCl was varied from 0.12 to 0.21 M and $CaCl_2$ was added at 0 or 1.0 mM.

Scanning electron microscopy

For the scanning electron microscopy, samples were prepared as described before [20], with a few minor modifications. Briefly, the final concentration of NaCl was 0.15 M in the absence of $CaCl_2$. Images were recorded at 3000x or 20000x magnification. Fifty fiber diameters for each clot were measured using a vernier caliper on a 300%

enlargement from a photograph made at 20000x magnification.

Statistical analysis

Statistical values for the comparisons between normal control and variant fibrinogen were determined using unpaired *t*-tests. A difference was considered significant when the *P*-value was <0.05.

Results

Coagulation tests and characterization of purified fibrinogen

Coagulation test results from the propositus and his mother and father are shown in Table 1. The plasma fibrinogen concentrations of the propositus were 0.89 g/l and 1.06 g/l, when functionally determined using Thrombocheck Fib-L and Dade Thrombin Reagent, respectively, and these concentrations were much lower than that determined by the immunological method, 2.41 g/l. On the other hand, that determined using MDA Fibriquik was 1.56 g/l, which was also lower than the latter. From the assay for the plasma fibrinogen concentrations of the propositus' mother and father we speculated that the propositus' mother's fibrinogen might have similar functional abnormality to the propositus' fibrinogen (Table 1). Analysis of purified plasma fibrinogen by SDS-PAGE under reducing conditions revealed that the fibrinogens from the propositus' mother and a healthy control subject were pure and that both had the normal Aα-, Bβ- and γ-chain components (not shown).

Table 1. Coagulation screening tests results

	proband	mother	father	reference range
PT(sec)	NT	12.8	11.8	11.5 - 15.0
APTT(sec)	NT	32.1	33.5	25.5 - 39.8
Functional fibrinogen (g/l)				
Thrombocheck Fib-L	0.89(0.37)*	1.59(0.50)*	2.44(0.79)*	1.60 - 3.70*
Dade Thrombin Reagent	1.06(0.44)	1.82(0.57)	2.58(0.84)	1.80 - 3.50
MDA Fibriquik	1.56(0.65)	2.43(0.76)	2.48(0.81)	1.80 - 3.50

Immunologic fibrinogen (g/l)	2.41	3.18	3.07	1.80 - 3.50

Numbers in parentheses are the ratio to the immunologic value.
Propositus' plasma (2 months after birth) was measured in Shinshu University Hospital and * was measured in Kyoto University Hospital.

Nucleotide-sequence analyses of the fibrinogen gene

An aberrant pattern was found in the sequencing-fluorogram of the PCR-amplified exon III of the Bβ-chain gene from the propositus and his mother (Fig. 1). We subcloned the DNA fragments and found a three-nucleotide deletion at positions 3590-3592 in the Bβ-chain gene (all nucleotide positions were numbered taking the starting point of the transcription of the Bβ-chain gene as 1) (GenBank accession number M64983) of some clones (Fig.1; sequenced using reverse primer), indicative of a heterozygous deletion of CCT (as read using forward primer). Such a nucleotide deletion results in the deletion of the Bβ111Ser amino acid residue (Fig.1). The father and the normal control did not show a three-nucleotide deletion at this position (Fig.1).

In other regions, for the propositus we found three silent mutations at residues 112Ser, 159Ser and 345Tyr in the Bβ-chain, namely, heterozygous TCC/TCT, homozygous AGC->AGT, and heterozygous TAC/TAT, respectively. For his mother we found at these residues heterozygous TCC/TCT, heterozygous AGC/AGT, and homozygous TAC, respectively, and for his father homozygous TCT, homozygous AGT, and heterozygous TAC/TAT, respectively. The amino acid at known polymorphic position Bβ448 was Lys, Arg/Lys, and Lys for the propositus, his mother, and father, respectively [21].

Functional analyses of purified fibrinogen from the propositus' mother

Thrombin-catalyzed fibrin polymerization was monitored as the change in turbidity at 350 nm. Representative turbidity curves for fibrinogen are shown in Figs.2A and 2B. We measured the lag period, which reflects the rate of protofibril formation, the maximum slope (Vmax), which reflects the rate of protofibril assembly into fibers (i.e., the lateral aggregation of protofibrils) and Δabsorbance over 30-min or 3-h. The Kyoto IV fibrinogen showed augmented thrombin-catalyzed fibrin polymerization compared

to normal fibrinogen in polymerization buffer containing 0.12 M NaCl with or without 1 mM Ca ion (Fig.2A). To confirm the above augmented polymerization of the Kyoto IV fibrinogen we performed a fibrin polymerization for another normal plasma fibrinogen, resulting in almost similar polymerization curves to the normal control fibrinogen (data not shown).

If the above-described thrombin-catalyzed fibrin polymerization curve was right, the Kyoto IV fibrinogen concentration determined by the thrombin-time method would be larger than that for normal control fibrinogen. Thus we speculated that the marked differences of the propositus' plasma fibrinogen concentrations determined using the three sets of reagents and analyzers might be caused by the difference of NaCl concentration contained in the reagents (Table 2). Therefore we performed polymerization while varying the NaCl concentration of the buffer from 0.12 M to 0.21 M in the absence of Ca ion (Fig2B), and analyzed the lag period, the maximum slope and Δabsorbance over 30-min or 3-h (Fig.3). At 0.12 M and 0.15 M NaCl, the lag period of Kyoto IV fibrinogen was similar to that of control fibrinogen; however, at 0.18 M and 0.21 M NaCl, the lag period of Kyoto IV fibrinogen was increased compared to that of control fibrinogen in a NaCl concentration-dependent manner. Interestingly, the maximum slope and Δabsorbance of Kyoto IV fibrinogen were both larger than those of control fibrinogen at NaCl concentrations from 0.12 M to 0.18 M, while the former was smaller than and the latter was similar to that of control fibrinogen at a NaCl concentration of 0.21 M.

Table 2. Comparison of fibrinogen assay reagents and assay values based on thrombin time method

parameter	fibrinogen assay reagent used in thrombin time method		
	MDA Fibriquik	DadeThrombin Reagent	Thrombocheck Fib-L
kind of plasma dilution buffer	imidazol	veronal	HEPES
plasma dilution (fold)	5	10	10
concentration of buffer (mM)	51	28.5	ND
pH of buffer	7.3	7.35	7.35
NaCl concentration of buffer (mM)	103	125	127
thrombin reagent			
origin of thrombin	bovine	bovine	human
concentration of thrombin (NIH U/ml)	100	100	200
kind of buffer	imidazol	veronal	acetic acid-Na acetate
pH of buffer	7.3	7.35	5.75
Ca concentration (mM)	0	0	25
NaCl concentration of reagent (mM)	140	125	270
final NaCl concentration (mM) derived from reagent	115	125	175
final Ca concentration (mM) derived from reagent	0	0	8.3
assay value of fibrinogen (g/l)	1.56	1.06	0.89
fibrinogen concentration ratio (functional/immunologic)	0.65	0.44	0.37

ND: not determined. HEPES: see text.

Scanning electron microscopy of fibrin clots and fibers

To examine the differences in the ultrastructure of the fibrin clot between the propositus' mother and the normal control, we observed the fibrin clots under a scanning electron microscope. The clots prepared from the variant fibrinogen with the

aid of thrombin were slightly different in ultrastructure from those prepared from normal fibrinogen, the density of the bundles of fibrin fibers being less in the former than in the latter (Figs.4A and 4B) and the fiber diameter being thicker in the former (95.9 ± 21.7 nm) than in the latter (76.4 ± 14.7 nm) (n=50) (p<0.001) (Figs.4C and 4D).

Discussion

We identified a novel variant fibrinogen, BβΔ111Ser, and designated it as Kyoto IV according to the place of residence of the propositus. The residue Bβ111Ser is located at the 31st residue numbering from the N-terminal disulfide ring, Bβ80Cys, and a point marking about the first one-third of the coiled-coil region and adjacent to the plasmin-sensitive segment, which represents a hinge about which the molecule adopts different conformations. Although 87 dysfunctional fibrinogen variants in 246 families have been reported, only 6 mutations in 7 families have been reported within the coiled-coil region: Aα-chain; AαΔ80Asn and Aα141Arg->Ser [6-8], Bβ-chain; Bβ148Lys->Asn, Bβ160Asn->Ser and Bβ166Arg->Cys [9-12], and γ-chain; γ82Ala->Gly [13,14].

Surprisingly, purified Kyoto IV fibrinogen showed augmented thrombin-catalyzed fibrin polymerization in polymerization buffer containing 20 mM HEPES, 0.12 M NaCl and 1 mM Ca ion (our regular buffer conditions). Namely, its maximum slope and Δabsorbance were higher than those obtained from normal fibrinogen, and thicker fibrin fibers were formed as compared with those of the normal control. Moreover, in the absence of Ca ion, Kyoto IV fibrinogen also showed augmented thrombin-catalyzed fibrin polymerization. Three variants reported in the coiled-coil region, Aα141Arg->Ser [7.8], Bβ160Asn->Ser [10] and Bβ166Arg->Cys [11,12], whose fibrinogen was associated with extra glycosylation at Aα139Asn, at Bβ158Asn and with disulfide-linked dimers or cysteine at the neo Cys residue, respectively, showed markedly impaired fibrin polymerization and formed thinner fibrin fibers in comparison with the normal control. These features were different from those of the fibrin polymerization and fiber diameter of BβΔ111Ser fibrinogen. In the fibrin polymerization of the former variants, the lateral aggregation was decreased due to interference by the repulsive forces generated by the negative electric charge of the

extra sialic acids or steric hindrance induced by the extra glycosylation, or by the disulfide-linked cysteine at the neo Cys residue. By contrast, purified fibrinogen from heterozygous AαΔ80Asn patient's plasma had a similar polymerization curve to the normal control (using polymerization buffer: 50 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$) and formed clot with slightly thicker fibrin fibers (mean of 600 fibers) in comparison with the normal control, however, the patient's clot had some regions composed of tightly packed thin fiber and a wide distribution of fiber diameters [6]. Based on these facts we think that AαΔ80Asn showed almost similar function to that of BβΔ111Ser rather than to those of Aα141Arg->Ser, Bβ160Asn->Ser or Bβ166Arg->Cys. On the other hand, Bβ148Lys->Asn fibrinogen might have normal function in fibrin polymerization, as indicated by its values in functional fibrinogen determination and calculated fibrinogen level from fibrinopeptide release [9]. One other variant, γ82Ala->Gly, showed reduced expression of the variant chain in plasma caused by aberrant splicing, which would produce an abnormal chain truncated after residue 97; however, the function of this variant fibrinogen in fibrin polymerization has not been analyzed yet [13,14].

Stasio ED et al. concluded that Cl^- is a basic physiological modulator of fibrin polymerization and acts to prevent the growth of thicker, stiffer, and straighter fibers by increasing the pKa of a basic group, however, they didn't find the structural domains responsible for Cl^- binding site of fibrin [22]. We indicated under normal physiological conditions Kyoto IV fibrinogen augmented the polymerization, but in 0.21 M NaCl it abruptly impaired polymerization compared with normal control. Therefore these results indicate that the coiled-coil region nearby the Bβ111Ser might be related to one of the candidates for Cl^- binding site(s) and modulate the lateral aggregation.

The Kyoto IV propositus and his mother showed no history of thrombosis or bleeding tendency. From our polymerization results we speculate that the BβΔ111Ser variant fibrinogen may not cause bleeding tendency in patients. Namely, BβΔ111Ser fibrinogen is polymerized increasingly by thrombin produced in the coagulation cascade in vivo because the plasma ionic strength corresponds to 0.15 M NaCl and plasma also contains 1 mM Ca ion. We demonstrated that Kyoto IV fibrinogen polymerized normally under the condition of 0.15 M NaCl and the polymerization was

further enhanced in the presence of Ca ion. The unusual NaCl dependency that we observed for the thrombin-catalyzed fibrin polymerization of Kyoto IV fibrinogen may partially explain some of the marked discrepancies (Thrombocheck Fib-L) of the functionally determined fibrinogen values among reagents and clinical laboratories. On the other hand, we can not explain the discrepancy of functionally determined fibrinogen concentrations between MDA Fibriquik and DadeThrombin Reagent simply by NaCl dependency. A functional plasma fibrinogen determination assay using the thrombin-time method was examined and established by Clauss (the Clauss method) [23]. The original method used the following buffer for dilution of plasma and thrombin: 28.5 mM sodium veronal, 125.5 mM NaCl and pH adjusted to 7.35 with HCl. However, recently most clinical laboratories use a set of commercially available ready-made kits and automated coagulation analyzers for the Clauss method. Most manufacturers prepare their kits with some modifications of the original reagents, resulting in a wide variety of constituents for reagents, with various kinds of buffer and buffer concentrations and NaCl concentrations (see Table 2). Although the original Clauss method does not precisely reflect the *in vivo* fibrinogen function as fibrin conversion by thrombin, we hope that the Clauss method will be standardized making it possible to obtain standardized values for variant fibrinogen such as Kyoto IV. In any case, when we encounter the hypofibrinogenemia in fibrinogen determination by functional method, immunologic (or antigenic) determination of fibrinogen is recommended for differential diagnosis among heterozygous or homozygous dysfibrinogenemia, heterozygous fibrinogen deficiency or other acquired hypofibrinogenemia.

In conclusion, we reported a novel heterozygous variant fibrinogen caused by a 3-bp deletion, BβΔ111Ser, and designated it as Kyoto IV. Functional determination based on the Clauss method showed discrepancies of the values obtained using three sets of reagents and analyzers. However, under normal physiological conditions for thrombin-catalyzed fibrin polymerization, the polymerization of Kyoto IV fibrinogen was augmented and thicker fiber clots were formed as compared with the normal control. The present study indicates that the residue located in coiled-coil region, Bβ111Ser, plays an important role in the lateral aggregation of protofibrils.

Acknowledgments

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List of abbreviations

PT: prothrombin time, APTT: activated partial thromboplastin time,

HEPES: N-[2-hydroxyethyl] piperazine-N'-[2- ethanesulfonic acid]

PCR: polymerase chain reaction, Vmax: maximum slope.

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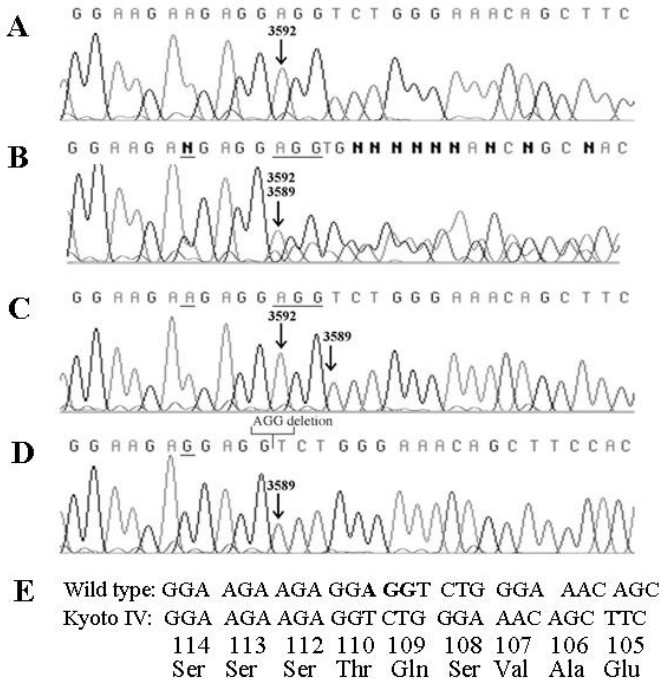

Figure 1. Nucleotide sequence of the fibrinogen Bβ-chain gene exon III. The PCR-amplified Bβ-chain genes of the normal control (A) and the propositus (B) were directly sequenced by the dideoxy termination method using reverse primer. Subcloned PCR-amplified Bβ-chain genes of the propositus were sequenced similarly. The nucleotides numbered from 3590 to 3592 were heterozygously deleted in the propositus' gene and the figure shows the data for the wild type (C) and variant type (D) of the cloned fragment. This three-nucleotide deletion leads to the deletion of amino acid Bβ111Ser (E).

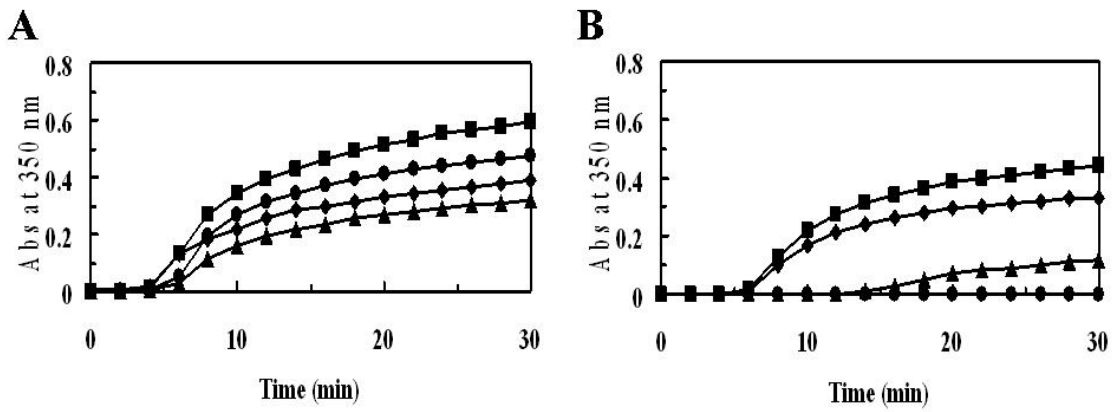

Figure 2. Thrombin-catalyzed fibrin polymerization. Thrombin (0.05 U/ml) was added at 0 min to fibrinogen (0.18 mg/ml) in 20 mM HEPES, pH 7.4, containing 0.12 M NaCl and with or without 1 mM $CaCl_2$ (A). Change in turbidity with time was followed at 350 nm. Representative polymerization curves from triplicate experiments (A) are shown for normal control (♦) and Kyoto IV (■) fibrinogen with 1mM $CaCl_2$, and for normal control (▲) and Kyoto IV (●) fibrinogen without $CaCl_2$. Thrombin-catalyzed fibrin polymerization was performed as described above except that the NaCl concentration in the buffer was 0.15 M or 0.21 M, and the reaction was performed without $CaCl_2$ (B). Representative polymerization curves from triplicate experiments (B) are shown for the normal control (♦) and Kyoto IV (■) fibrinogen with 0.15 M NaCl and for the normal control (▲) and Kyoto IV (●) fibrinogen with 0.21 M NaCl.

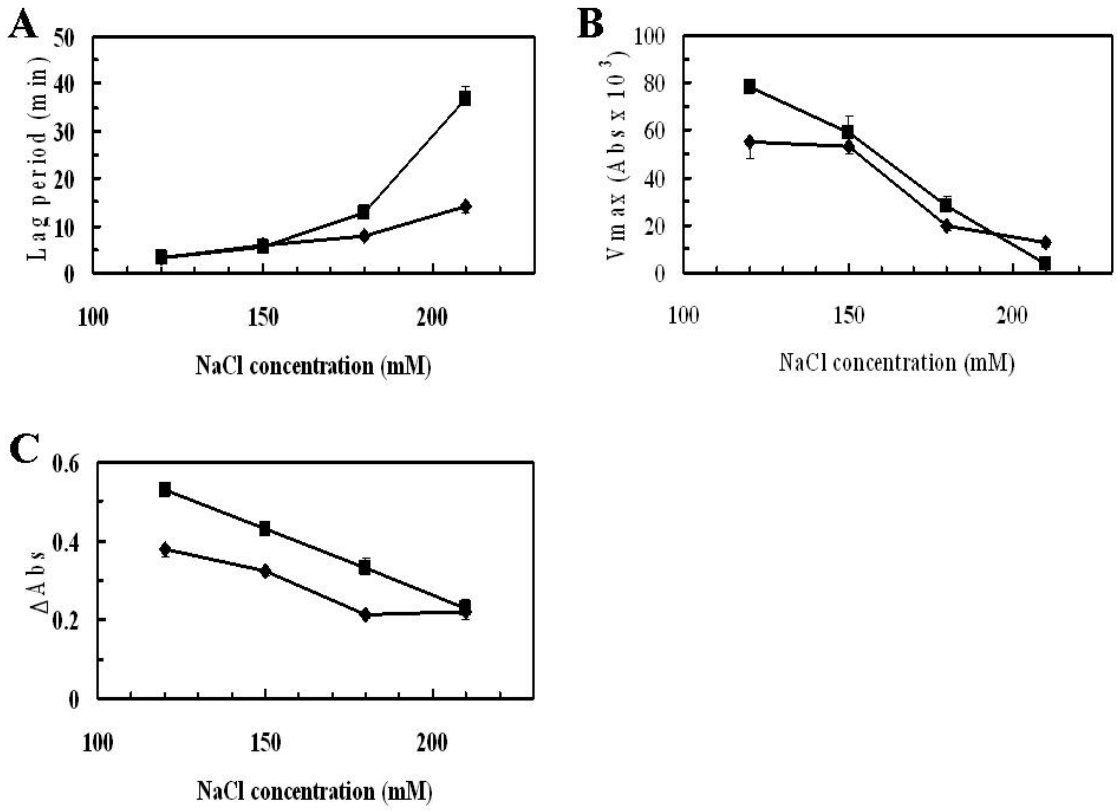

Figure 3. Dependency of three parameters of thrombin-catalyzed fibrin polymerization on NaCl concentration in buffer. The lag period (A), which reflects the rate of protofibril formation, the maximum slope (Vmax) (B), which reflects the rate of protofibril assembly into fibers (i.e., the lateral aggregation of protofibrils) and Δabsorbance over 30-min (at 0.12 M-0.18 M NaCl) or 3-h (at 0.21 M NaCl) (C), which reflects the fiber diameter, were plotted against the NaCl concentration in the buffer. The mean values and standard deviations for the normal control (♦) and Kyoto IV (■) fibrinogen from triplicate experiments are shown.

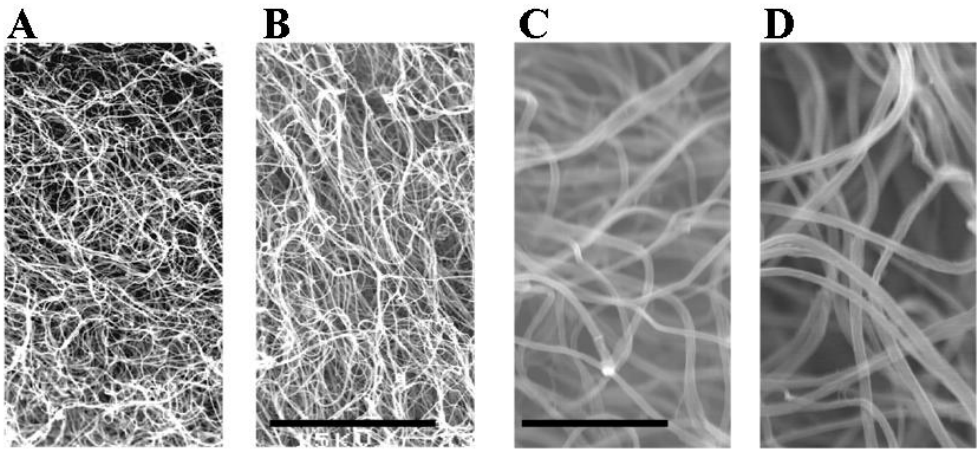

Figure 4. **Scanning electron microscopy of fibrin clots formed using thrombin.** Clots were polymerized, fixed, stained, critical-point dried, mounted, and osmium plasma-coated. Fibrinogen (0.18 mg/ml) was polymerized using 0.1 U/ml of thrombin. Photomicrographs of fibrin clots made from normal control (A, C) and Kyoto IV (B, D) fibrinogen were taken at 3,000-(A,B) or 20,000-(C,D) fold magnification. The bar represents 10 μm in B and 1 μm in C.